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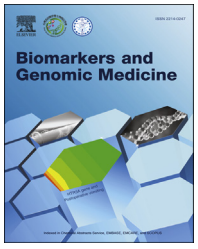

SHORT COMMUNICATION

Mutations in the quinolone resistance-determining regions associated with ciprofloxacin resistance in *Pseudomonas aeruginosa* isolates from Southern Taiwan

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Abstract Mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* have been characterized among the 232 isolates of ciprofloxacin (CIP)-resistant *Pseudomonas aeruginosa*. As expected, no mutations in the QRDRs of four target genes were detected in the CIP-susceptible isolates of *P. aeruginosa*. It was noted that *P. aeruginosa* showing no mutation in the QRDRs of target genes were frequently found in isolates with a CIP in minimal inhibitory concentration (MIC) = 2 μg/mL than those of isolates with a CIP in MIC ≥4 μg/mL. The prevalence of *P. aeruginosa* with no mutations in the QRDRs of target genes is higher in isolates only resistant to CIP than in isolates resistant to CIP and other drugs. Double mutations occurring in *gyrA* and *parC* genes associated with a high-level resistance to CIP in MICs ≥4 μg/mL were found in 101 out of 176 isolates. Furthermore, mutations in *parC* and *parE* joined with mutation in *gyrA* were commonly found in *P. aeruginosa* highly resistant to CIP.

Introduction

Because of the possession of a high level in intrinsic and acquired resistance, therapeutic challenge is of great concern in the treatment of infectious diseases caused by multidrug-resistant *Pseudomonas aeruginosa*.[1] Current antibiotics used for multidrug-resistant *P. aeruginosa* infections are limited to fluoroquinolones, aminoglycosides, and carbapenems. Ciprofloxacin (CIP) has been shown to be effective in the treatment of serious infections with *P.*

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aeruginosa. It showed a rapid bactericidal effect against the dual targets of DNA gyrase and topoisomerase IV.[2] Bacterial DNA gyrase and topoisomerase IV are the major targets of quinolone antimicrobial agents. Therefore, mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* genes were found to be associated with fluoroquinolone resistance in most bacterial species including *P. aeruginosa*.[3,4] In Taiwan, *P. aeruginosa* showed 6.2—16.1% resistance to CIP.[5] As regards resistance to CIP associated with target gene mutations in the QRDRs, little information is known about *P. aeruginosa* in Taiwan. The aim of this study is to demonstrate the molecular mechanism of CIP resistance in *P. aeruginosa* due to target gene mutations. The point mutations involved in the QRDRs of DNA gyrase in *gyrA* and *gyrB* genes and topoisomerase IV in *parC* and *parE* genes among CIP-resistant *P. aeruginosa* isolates were also characterized.

Materials and methods

Bacterial strain

In this study, a total of 262 nonduplicate clinical isolates of *P. aeruginosa* used to screen mutations in the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* genes, including 30 isolates susceptible to CIP (MICs of $\leq$1 μg/mL), 56 isolates intermediate-resistant to CIP, with MIC = 2 μg/mL, and 176 isolates highly resistant to CIP, with MICs ranging from 4 μg/mL to $\geq$125 μg/mL. Among the 232 isolates of CIP-resistant *P. aeruginosa*, 48 isolates showed resistance to CIP only, and the remaining 184 isolates were simultaneously resistant to CIP and other drugs such as ceftazidime, meropenem, gentamicin, amikacin, or piperacillin. These isolates were obtained from Kaohsiung Medical University Hospital, Kaohsiung, Taiwan in 2010—2012. The antibiotic susceptibility test of minimal inhibitory concentrations (MICs) for all isolates was conducted using the VITEK 2 system (bioMérieux, Marcy l'Etoile, France).

Polymerase chain reaction techniques and BLAST program

Bacterial genomic DNA was extracted using a Genomic DNA Purification Kit (Promega Biosciences Inc., San Luis Obispo, CA, USA). All isolates of *P. aeruginosa* were screened for *gyrA*, *gyrB*, *parC*, and *parE* genes by polymerase chain reaction (PCR) amplification using specific primers against bacterial DNA template as previously described.[6,7] Primers gyrA-F (5′-AGTCCTATCTCGAC TACGCGAT-3′) and gyrA-R (5′-AGTC GACGGTTTCCTTTTCCAG-3′) were used to amplify 324 bp of the *gyrA* gene (accession no. AAG06556). Primers gyrB-F (5′-TGCGGTGGAACAGG AGATGGGCAAGTAC-3′) and gyrB-R (5′-CTGGCGGAAGAAGAAGGTCAAC AGCAGG GT-3′) were used to amplify 483 bp of the *gyrB* gene (accession no. AAG03394). Primers parC-F (5′-CGAGCAGGCCTATCTGAACTAT-3′) and parC-R (5′-GAAGGACTTGGGATC GTCCGGA-3′) were used to amplify 282 bp of the *parC* gene (accession no. AAG08349). Primers parE-F (5′-CGGCGTTCGTCTCGGGCGTGGTGAAGGA-3′) and parE-R (5′-TCGAGG GCGTAGTAGATGTCCTTGCCGA-3′) were used to amplify 564 bp of the *parE* gene (accession no. AAG08352). The amplification techniques were performed using Fast-Run Taq TM Master kit (Protech Technology Enterprises Co., Ltd, Taipei, Taiwan). The PCR reactions were performed on a DNA thermalcycler (Gradient master cycler; Eppendorf, USA) for 35 cycles, each cycle consisting of 1 minute at 94°C for denaturation, 30 seconds at 55°C (for *gyrA* and *parC* genes) or 60°C (for *gyrB* and *parE* genes) for annealing, and 1 minute at 72°C for polymerization. The PCR products were purified with a PCR-M Clean up System Kit (Viogene, Sunnyvale, CA, USA) and then directly sequenced on both strands by means of the primer walking sequence strategy. The DNA sequencing analysis was compared with those available in the sequence of wild-type *P. aeruginosa* PAO1 (accession no. NC-002516) at GenBank data using the BLAST suite of programs (http://www.ncbi.nlm.nih.gov/BLAST).

Results

The relationship between mutations of QRDRs target genes and antibiotic resistance patterns in *P. aeruginosa* isolates are listed in Table 1. A total of seven types of mutation in QRDRs target genes were detected in CIP-resistant *P. aeruginosa* isolates. In general, double mutations of *gyrA—parC* genes (Type I) were the most frequently found in CIP-resistant *P. aeruginosa* isolates tested (43.1%, 100/232). The majority of CIP-resistant *P. aeruginosa* isolates (76.7%, 178/232) showed single mutation in *gyrA* gene (Types I, II, III, and V). Mutations in *gyrB* gene (Type IV) were only found in 10 (4.3%) out of 232 CIP-resistant *P. aeruginosa*. Single *parC* mutation (Type VI) was also only found in 1.1% (2/184) of *P. aeruginosa* isolates resistant to CIP together with other selected drugs, but did not occur in isolates only resistant to CIP.

The mutation frequency of the QRDR target genes in *P. aeruginosa* isolates showing resistance to CIP together with other drugs (79.3%, 184/232) was higher than that in isolates resistant only to CIP (20.7%, 48/232). The former isolates showed a higher incidence (51.1%, 94/184) of Type I QRDR mutations of *gyrA—parC* genes than that of the latter isolates (12.5%, 6/48). Isolates of CIP-resistant *P. aeruginosa* showing no mutations in QRDR target genes were categorized as Type VII QRDR mutations. Among the Type VII QRDR mutations, the percentage of *P. aeruginosa* isolates showing resistance only to CIP (37.5%, 18/48) was higher than that of isolates resistant to CIP together with other drugs (13.0%, 24/184). Furthermore, mutation in QRDR target genes of *gyrA*, *gyrB*, *parC*, and p*arE* does not occur simultaneously in the *P. aeruginosa* isolates tested. From our results, mutations of QRDRs were not found in the sequence area in *gyrA*, *gyrB*, *parC*, and *parE* genes among these 30 CIP-susceptible (MICs $\leq$1 μg/mL) *P. aeruginosa* isolates (data not shown).

The mutation of QRDR target genes leading to amino acid substitutions sorted by CIP in MIC values for *P. aeruginosa* isolates are listed in Table 2. A significant difference was detected in the frequency of QRDR mutations among CIP-resistant *P. aeruginosa* between high-level resistant isolates (MIC $\geq$ 4 μg/mL; 75.8%, 176/232) and low-level resistant isolates (MIC = 2 μg/mL; 24.1%, 56/232). With respect to Type I QRDR mutation, double mutations of

Table 1 The relationship between mutations of the quinolone resistance-determining regions and antibiotic resistance patterns in *Pseudomonas aeruginosa* isolates.

Type of mutation	Mutations of				Antibiotic resistance patterns			Total (%)
	DNA gyrase		Topoisomerase IV		Resistant to CIP only	Multidrug-resistant to CIP and other drugs selected	*p*	
	gyrA	*gyrB*	*parC*	*parE*	No. (%)	No. (%)		
Type I	+	−	+	−	6 (12.5)	94 (51.1)	<0.01	100 (43.1)
Type III	+	−	−	−	18 (37.5)	40 (17.2)	<0.05	58 (25.0)
Type IV	+	−	−	+	4 (8.3)	12 (6.5)	NS	16 (6.9)
Type V	−	+	−	−	2 (4.2)	8 (4.3)	NS	10 (4.3)
Type VI	+	−	+	+	0	4 (2.2)	NS	4 (1.7)
Type VII	−	−	+	−	0	2 (1.1)	NS	2 (0.09)
Type II	−	−	−	−	18 (37.5)	24 (13.0)	<0.05	42 (18.1)
Total (%)					48 (20.7)	184 (79.3)	<0.01	232

Statistical significance (*p*) was calculated using the Pearson Chi-square test in terms of the number of strains.
+ = mutation; − = no mutation; CIP = ciprofloxacin; NS = not statistically significant.

gyrA—*parC* genes associated with variable amino acid substitutions were mostly responsible for the high-level resistance to CIP (43.1%, 100/232). The *gyrA*—*parC* mutation was commonly found in isolates high-level resistant to CIP (52.8%, 93/176) than in isolates low-level resistant to CIP (10.7%, 6/56). It was apparent that *P. aeruginosa* with no mutation in QRDR target genes (Type VII) was frequently found in isolates low-level resistant to CIP (42.9%, 24/56) than in isolates high-level resistant to CIP (10.2%, 18/176).

The most prevalent mutation in the *gyrA* gene was the Thr83Ile substitution among isolates harboring *gyrA*—*parC* mutation (Type I QRDR mutation; 99.0%, 98/99). By contrast, variable amino acid substitutions in the *parC* gene with Gly85Cys, Ser87Leu, Ser87Trp, and Glu91Lys were found. Single g*yrA* mutations in isolates of Type II QRDR mutation were frequently identified at amino acid Thr83Ile substitution (74.1%, 40/54). However, a variety of amino acid substitutions occurring at position 87 in g*yrA* mutations with Asp87Asn, Asp87His, Asp87Tyr, and Asp87Gly were found. There was no remarkable difference in mutation frequency in the *gyrA* gene among *P. aeruginosa* between isolates low-level resistant to CIP (32.1%, 18/56) and isolates high-level resistant to CIP (21.0%, 37/176). The Type III QRDR mutation in *gyrA*—*parE* genes was more frequently found in *P. aeruginosa* isolates with a CIP in MIC $\geq$4 μg/mL (93.7%, 15/16) than in isolates intermediate-resistant to CIP (MIC = 2 μg/mL; 6.3%, 1/16). Among the isolates with Type III QRDR mutation of *gyrA*—*parE* genes, the *parE* gene showed a highly variable locus within the amino acid substitutions, and most of the *gyrA* also showed a Thr83Ile substitution (93.7%, 15/16).

Among the Type IV QRDR mutations of the *gyrB* gene, all 10 isolates had only one type mutation of Ser466Phe substitution. These isolates with Type-IV QRDR mutation were frequently associated with high-level resistance to CIP (80%, 8/10). Only four out of 232 isolates (1.8%) belonged to Type V QRDR mutation of *gyrA*—*parC*—*parE* genes. It exhibited two type mutations of g*yrA* (Thr83Ile)—*parC* (Ser87Leu)—*parE* (Ser457Arg) and g*yrA* (Thr83Ile)—*parC* (Ser87Leu)—*parE* (Glu459Val). Finally, there are only two isolates harboring Type VI QRDR mutation in the *parC* gene. However, these two isolates have the same mutation of the *parC* gene at Ser87Leu with a MIC $\geq$4 μg/mL.

Discussion

Our results indicate that these four QRDR target genes of DNA gyrase and topoisomerase IV are highly associated with resistance to CIP in *P. aeruginosa* isolates. However, *P. aeruginosa* isolates showing no mutation in QRDR target genes was more frequently found in lower CIP-resistant isolates than those in higher CIP-resistant isolates. These results suggested that low-level resistance to CIP in *P. aeruginosa* isolates is associated with target gene mutations in QRDRs and other additional different efflux pump systems.[8,9] Moreover, CIP-resistant *P. aeruginosa* isolates showing mutations in QRDR target genes were less frequently found in isolates resistant only to CIP than in isolates resistant to CIP together with other drugs such as ceftazidime, meropenem, gentamicin, amikacin, or piperacillin. These results suggested that mutations within *gyrA*, *gyrB*, *parC*, and *parE* genes were associated with resistance not only to CIP but also to other selected drugs.[10]

It was noted that CIP-resistant *P. aeruginosa* isolates showed the highest frequency of mutation in the *gyrA* gene among these four QRDR target genes. Although mutations in *gyrA*—*parC*, *gyrA*—*parE*, and *gyrA*—*parC*—*parE*, individually, associated with high-level resistance to CIP, a single mutation in *gyrA* was responsible for the variable MIC values of resistance to CIP in *P. aeruginosa* isolates.[11] In other studies, 98.1% of mutation at Thr83Ile occurring in the *gyrA* gene, and double mutations in the *gyrA* gene have been described at Thr83Ile and Asp87Asn or Tyr.[7,11–13] In our isolates, *gyrA* gene mutation at Thr83Ile conferred high-level resistance in *P. aeruginosa* (MICs $\geq$ 4 μg/mL), and mutation in *gyrA* at the codon 87 position contributed to the low-level resistance to CIP (MIC = 2 μg/mL). CIP-resistant isolates carrying mutations in the *gyrB* gene at codons 464, 466, 468, and 475 positions have been demonstrated in other CIP-resistant

Table 2 The correlation of amino acid substitution in mutations of the quinolone resistance-determining regions and CIP resistance in MIC (μg/mL) among *Pseudomonas aeruginosa* isolates.

Type of mutation	Amino acid substitution of				No. of isolates resistant to CIP with MIC (μg/mL)			Total (%)
	DNA gyrase		Topoisomerase IV		2	≥4	Subtotal	
	gyrA	*gyrB*	*parC*	*parE*	No.	No.		
Type I	Thr83Ile	—	Ser87Leu	—	0	78	78	100 (43.1)
	Thr83Ile	—	Glu91Lys	—	3	7	10	
	Thr83Ile	—	Gly85Cys	—	2	4	6	
	Thr83Ile	—	Ser87Trp	—	0	3	3	
	Thr83Ile	—	Ser87Leu Gly85Cys	—	0	2	2	
	Asp87Tyr	—	Ser87Leu	—	1	0	1	
Type II	Thr83Ile	—	—	—	10	36	46	58 (25.0)
	Asp87Asn	—	—	—	4	3	7	
	Asp87His	—	—	—	3	0	3	
	Asp87Tyr	—	—	—	1	0	1	
	Asp87Gly	—	—	—	1	0	1	
Type III	Thr83Ile	—	—	Leu501Phe	0	3	3	16 (6.9)
	Thr83Ile	—	—	Ser457Gly	0	3	3	
	Thr83Ile	—	—	Glu453Asp	0	3	3	
	Thr83Ile	—	—	Asp419Asn	0	2	2	
	Thr83Ile	—	—	Lys388Glu	0	1	1	
	Thr83Ile	—	—	Glu459Val	0	2	2	
	Thr83Ile	—	—	Val460Phe Ala473Val	0	1	1	
	Asp87Asn	—	—	Ala473Thr	1	0	1	
Type IV	—	Ser466Phe	—	—	2	8	10	10 (4.3)
Type V	Thr83Ile	—	Ser87Leu	Ser457Arg		2	2	4 (1.7)
	Thr83Ile	—	Ser87Leu	Glu459Val		2	2	
Type VI	—	—	Ser87Leu	—	2	0	2	2 (0.09)
Type VII	—	—	—	—	24	18	42	42 (18.1)
Total					56	176	232	232

— = no mutation; CIP = ciprofloxacin; MIC = minimal inhibitory concentration.

isolates of *P. aeruginosa*.[10–13] However, single mutation in *gyrB* gene with the same amino acid substitution at Ser466Phe was detected in this study. Although single mutation in the *parC* gene encoded moderate resistance to CIP, *parC* mutation together with mutations in *gyrA* or in combination with *gyrA*–*parE* genes were commonly associated with high resistance to CIP in our *P. aeruginosa* isolates.[14] As described in previous reports, mutations in the *parE* gene for fluoroquinolone resistance was rare and showed highly variable profiles of amino acid substitutions.[11,15] Noticeably, three novel mutation sites at Lys388Glu, Leu501Phe, and Val460Ala in the *parE* gene are first reported in this study, and these three mutation sites mediated high-level resistance to CIP. Furthermore, mutations in *parE* linking simultaneously with mutation in *gyrA* or *gyrA*–*parC* would encode high-level resistance to CIP in *P. aeruginosa* isolates.

In conclusion, g*yrA* is the primary target gene in the QRDR mutation to mediate CIP resistance in *P. aeruginosa* isolates. Double mutations of *gyrA*–*parC* or *gyrA*–*parE*, and triple mutations of *gyrA*–*parC*–*parE* were mostly associated with high-level resistance to CIP in *P. aeruginosa* isolates. The diversity of mutations in *parC* and *parE* genes was more frequently found than that of g*yrA* and *gyrB* genes in our *P. aeruginosa* isolates.

Conflicts of interest

All authors declare no conflicts of interest.

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